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TruSight™ Rapid Capture Sample Preparation Guide

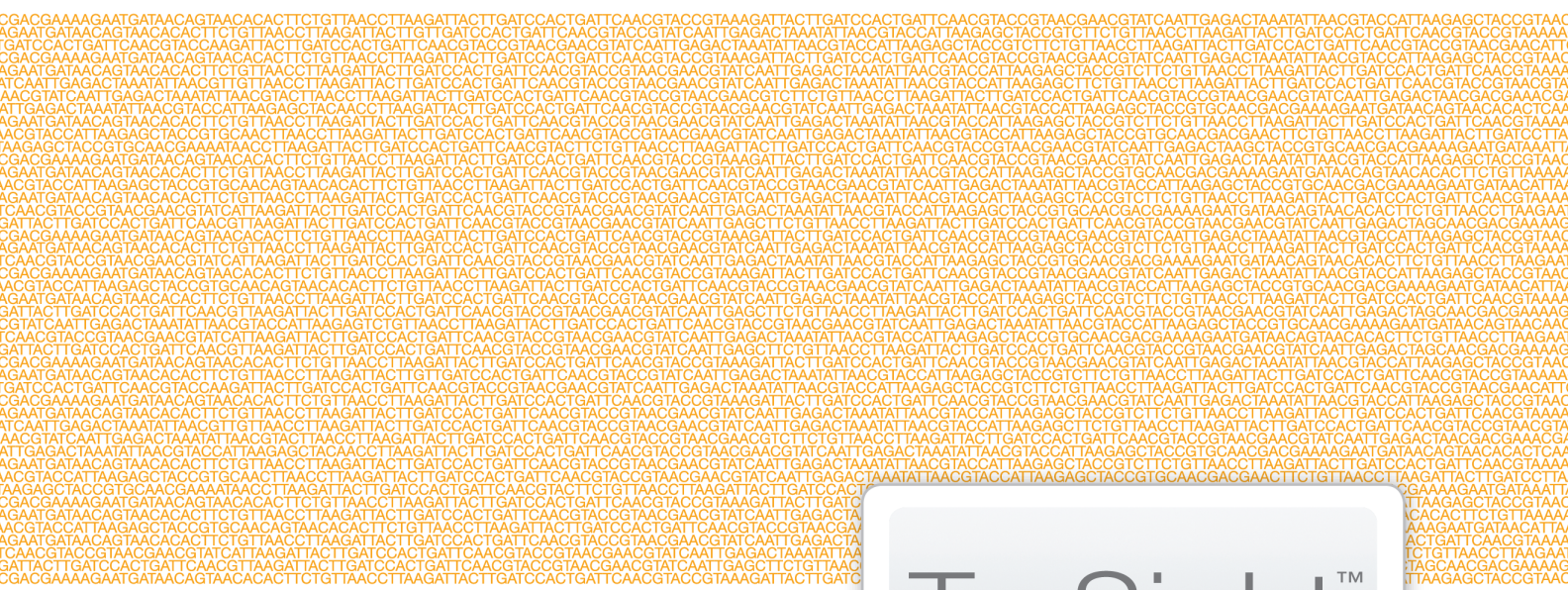

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Table of Contents

Overview

Chapter 1

Introduction

This protocol explains how to prepare up to 96 indexed, paired-end libraries, followed by enrichment using a TruSight™ Content Set and reagents provided by an Illumina TruSight Rapid Capture kit. The goal of this protocol is to fragment and add adapter sequences onto template DNA to generate indexed sequencing libraries that can be carried through enrichment for targeted resequencing applications.

The TruSight Rapid Capture protocol offers:

- Fast and easy sample preparation
  - Prepare up to 96 enriched libraries in approximately 1.5 days, including approximately 5 hours of hands-on time
  - High throughput, automation-friendly procedures with no fragmentation bottlenecks
- Low DNA input and excellent data quality
  - Excellent data quality with low input of 50 ng
  - Access precious samples with no impact to performance
  - Ability to archive samples for additional analysis
- High enrichment rates, low duplicates, and exceptional coverage uniformity
  - Efficient use of sequencing and reliable variant calling
  - Reduced hands-on time with the most cost-effective, high-throughput workflow

DNA Input Recommendations

TruSight Rapid Capture library preparation uses an enzymatic DNA fragmentation step and thus can be more sensitive to DNA input compared to mechanical fragmentation methods. The ultimate success of enrichment strongly depends on using an accurately quantified amount of input DNA. Therefore, accurate quantitation of the gDNA is essential.

Illumina recommends quantifying the starting gDNA using a fluorometric-based method specific for double-stranded DNA (dsDNA) such as QuantiFluor and running samples in triplicate to obtain more confident measurements. Methods that measure total nucleic acid content (e.g. nanodrop or other UV absorbance methods) should be avoided because common contaminants such as ssDNA, RNA, and oligos are not substrates for the TruSight Rapid Capture assay. For more information, see *DNA Quantitation* on page 68.

The TruSight Rapid Capture protocol has been optimized for 50 ng of total gDNA. A higher mass input of gDNA may result in incomplete tagmentation and larger insert sizes, which can impact enrichment performance. Conversely, a low mass input of gDNA or low quality gDNA in the tagmentation reaction may generate smaller than expected insert sizes, which can be lost during subsequent clean-up steps resulting in lower diversity.

To minimize gDNA sample input variability into the tagmentation step, Illumina strongly recommends a two-step method of gDNA normalization. After the initial quantification, gDNA samples are first normalized to 10 ng/ul. Samples are then re-quantified using a similar fluorometric-based method and normalized to a final 5 ng/ul.

Additional Resources

The following resources are available for TruSight Rapid Capture protocol guidance and sample tracking. Access these and other resources on the Illumina website at support.illumina.com/sequencing/kits.ilmn. Then, select **TruSight Rapid Capture Sample Prep Kit Support**.

Resource	Description
Best Practices	Provides best practices specific to this protocol. Review this before starting sample preparation. Topics include: • Consistency • Handling Magnetic Beads • Avoiding Cross-Contamination • Washing During SPB Clean-Up • Freeze/thawing for Small Number of Samples • Preventing PCR Product Contamination Click **Best Practices** on the **TruSight Rapid Capture Sample Prep Kit Support** page.
TruSight Rapid Capture Experienced User Card (part # 15043292)	Provides protocol instructions, but with less detail than what is provided in this user guide. **New or less experienced users are strongly advised to follow this user guide and not the EUC.** Click **Documentation & Literature** on the **TruSight Rapid Capture Sample Prep Kit Support** page.
Illumina Experiment Manager (IEM)	Enables you to create and edit appropriate sample sheets for Illumina sequencers and analysis software and record parameters for your sample plate. To download the software, click **Downloads** on the **TruSight Rapid Capture Sample Prep Kit Support** page. To download the documentation, click **Documentation & Literature** on the **TruSight Rapid Capture Sample Prep Kit Support** page.

Protocol

Chapter 2

Introduction

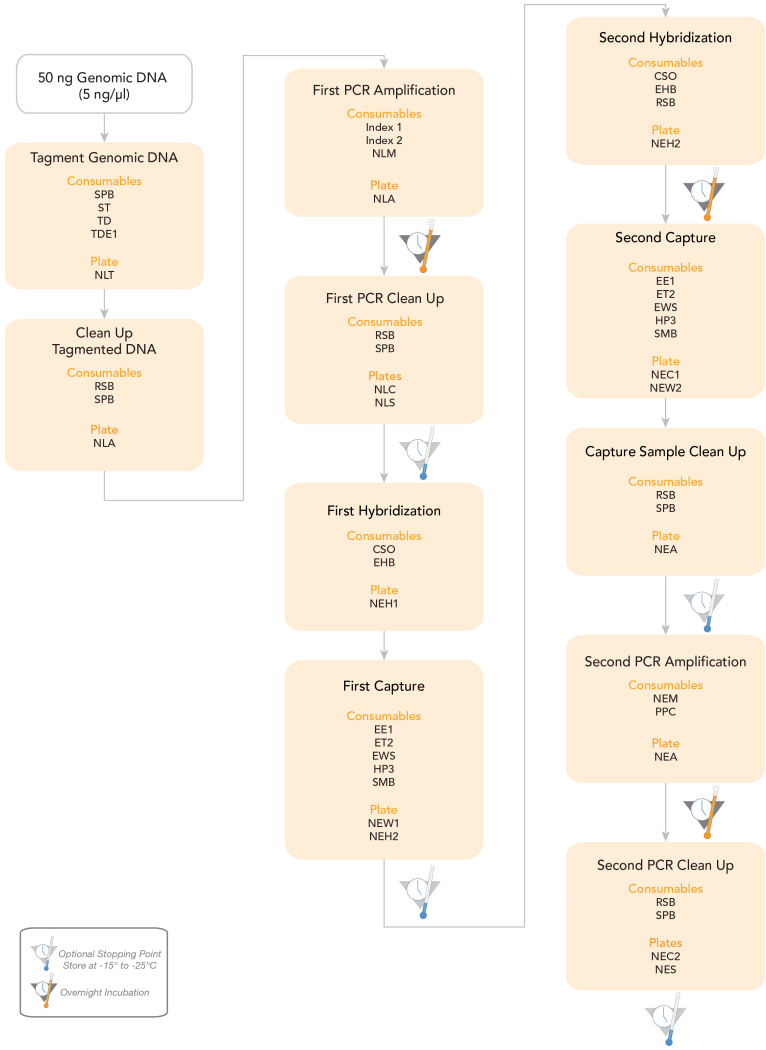
This chapter describes the TruSight Rapid Capture protocol.

- Review Best Practices before proceeding. See *Additional Resources* on page 4 for information on how to access TruSight Rapid Capture Best Practices on the Illumina website.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- If you are pooling, record information about your samples before beginning library preparation for later use in data analysis.
  - Use IEM to create and edit well formed sample sheets for Illumina sequencers and analysis software. See *Additional Resources* on page 4 for information on how to download IEM software and documentation from the Illumina website.
  - Each column should contain a common index. This will facilitate pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol. For more information, see *Low-Plex Pooling Guidelines* on page 65.

Sample PrepWorkflow

The following diagram illustrates the workflow using a TruSight Rapid Capture kit. Safe stopping points are marked between steps.

Figure 1 TruSight Rapid Capture Workflow



Tagment Genomic DNA

This process tagments (tags and fragments) the gDNA by the Nextera transposome. The Nextera transposome simultaneously fragments the gDNA and adds adapter sequences to the ends, allowing amplification by PCR in subsequent processes.

Consumables

Item	Quantity	Storage	Supplied By
Sample Purification Beads (SPB)	1 tube	2° to 8°C	Illumina
Stop Tagment Buffer (ST)	1 tube	15° to 30°C	Illumina
Tagment DNA Buffer (TD)	1 tube	-15° to -25°C	Illumina
Tagment DNA Enzyme (TDE1)	1 tube	-15° to -25°C	Illumina
96-well MIDI plate	1	15° to 30°C	User
gDNA (5 ng/µl)	50 ng	-15° to -25°C	User
Microseal 'B' adhesive seals	2	15° to 30°C	User
RNase/DNase-free Reagent Reservoir (for multi-sample processing)	1	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (for multi-sample processing)	3	15° to 30°C	User
Tris-Cl 10 mM, pH 8.5	as needed	15° to 30°C	User

Preparation

- Remove the Tagment DNA Buffer, Tagment DNA Enzyme 1, and gDNA from -15° to -25°C storage and thaw on ice.
  - After thawing, ensure all reagents are adequately mixed by gently inverting the tubes 3–5 times, followed by a brief spin in a microcentrifuge.

- Remove the Sample Purification Beads from 2° to 8°C storage and let stand to bring to room temperature.
- Make sure the Stop Tagment Buffer has no precipitate. If there is precipitate, vortex until all particulates are resuspended.
- For multi-sample processing:
  - Use a multichannel pipette.
  - Distribute the Stop Tagment Buffer, Tagment DNA Buffer, and Tagment DNA Enzyme 1 into separate eight-tube strips, dispensing equal volumes into each of the wells.
  - Pour the Sample Purification Beads into a multichannel reagent reservoir.
- Place a MIDI plate insert on the microheating system.
- Pre-heat the microheating system to 58°C.
- Label a new 96-well MIDI plate **NLT** (Nextera Library Tagment) with a smudge resistant pen.
- Use the Illumina Experiment Manager to determine the index primers to be used. For more information on IEM, see *Additional Resources* on page 4.

Procedure

NOTE
Ensure the reaction is assembled in the order described for optimal kit performance. The reaction does not need to be assembled on ice.

1. Perform the following steps to normalize your gDNA samples:
   a. Quantify your gDNA samples using a fluorometric method such as QuantiFluor or Qubit.
   b. Normalize your gDNA samples in Tris-Cl 10 mM, pH 8.5 to 10 ng/µl.
   c. Re-quantify the 10 ng/µl normalized sample using the same fluorometric quantification method.
   d. Based on this quantification, further dilute your gDNA samples in Tris-Cl 10 mM, pH 8.5 to a final volume of 10 µl at 5 ng/µl (50 ng total) for use in the tagmentation reaction.
2. Add 10 µl of gDNA at 5 ng/µl (50 ng total) to each well of the new 96-well MIDI plate labeled NLT.
3. Add 25 µl of Tagment DNA Buffer to each well of the NLT plate.
4. Add 15 µl of Tagment DNA Enzyme 1 to each well of the NLT plate.

5 Mix thoroughly as follows:
   a Seal the NLT plate with a Microseal 'B' adhesive seal.
   b Shake the NLT plate on a microplate shaker at 1800 rpm for 1 minute.

6 Centrifuge the NLT plate to 280 xg for 1 minute.

7 Place the sealed NLT plate on the pre-heated microheating system. Close the lid and incubate at 58°C for 10 minutes.

8 Remove the NLT plate from the microheating system.

9 Remove the adhesive seal from the NLT plate.

10 Add 15 µl Stop Tagment Buffer to each well of the NLT plate.

11 Mix thoroughly as follows:
   a Seal the NLT plate with a Microseal 'B' adhesive seal.
   b Shake the NLT plate on a microplate shaker at 1800 rpm for 1 minute.

12 Centrifuge the NLT plate to 280 xg for 1 minute.

13 Incubate the NLT plate at room temperature for 4 minutes.

14 Proceed to *Clean Up Tagmented DNA* on page 11.

Clean Up Tagmented DNA

This process purifies the tagmented DNA from the Nextera transposome. It is critical because the Nextera transposome can bind tightly to DNA ends and will interfere with downstream processes if not removed.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	-15° to -25°C	Illumina
Sample Purification Beads (SPB)	1 tube	2° to 8°C	Illumina
96-well hard-shell plate (HSP)	1	15° to 30°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15° to 30°C	User
Microseal 'B' adhesive seals	2	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (for multi-sample processing)	3	15° to 30°C	User

Preparation

- Remove the Resuspension Buffer from -15° to -25°C storage and thaw at room temperature.

NOTE
The Resuspension Buffer can be stored at 2° to 8°C after the initial thaw.

- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 4 for information on how to access TruSight Rapid Capture Best Practices on the Illumina website.
- Make sure that the Sample Purification Beads are at room temperature.
- For multi-sample processing:
  - Use a multichannel pipette.
  - Pour the Resuspension Buffer, Sample Purification Beads, and 80% EtOH into separate multichannel reagent reservoirs.

- Label a new 96-well HSP plate **NLA** (Nextera Library Amplification) with a smudge resistant pen.

Procedure

1. Remove the adhesive seal from the NLT plate.
2. Add 65 µl of well-resuspended Sample Purification Beads to each well of the NLT plate.
3. Mix thoroughly as follows:
   a. Seal the NLT plate with a Microseal 'B' adhesive seal.
   b. Shake the NLT plate on a microplate shaker at 1800 rpm for 1 minute.
4. Incubate the NLT plate at room temperature for 8 minutes.
5. Centrifuge the NLT plate to 280 xg for 1 minute.
6. Place the plate on the magnetic stand for 2 minutes or until the liquid appears clear.
7. Remove the adhesive seal from the NLT plate.
8. Using a 200 µl single channel or multichannel pipette set to 130 µl, remove and discard all of the supernatant from each well of the NLT plate.

   NOTE
   Leave the NLT plate on the magnetic stand while performing the following 80% EtOH wash steps (9–11).

9. With the NLT plate remaining on the magnetic stand, slowly add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads. Wait 30 seconds.
10. Remove and discard the 80% EtOH from each well of the NLT plate.
11. Repeat steps 9 and 10 once for a total of two 80% EtOH washes.
12. Using a 20 µl single channel or multichannel pipette, remove any remaining 80% EtOH from each well of the NLT plate without disturbing the beads.
13. With the NLT plate remaining on the magnetic stand, incubate the plate at room temperature for 10 minutes to dry.
14. Remove the NLT plate from the magnetic stand.
15. Add 22.5 µl of Resuspension Buffer to each well of the NLT plate. Do not touch the beads with the pipette tips.

16 Mix thoroughly as follows:
   a Seal the NLT plate with a Microseal 'B' adhesive seal.
   b Shake the NLT plate on a microplate shaker at 1800 rpm for 1 minute

17 Incubate the NLT plate at room temperature for 2 minutes.

18 Centrifuge the NLT plate to 280 xg for 1 minute.

19 Place the NLT plate on the magnetic stand for 2 minutes or until the liquid appears clear.

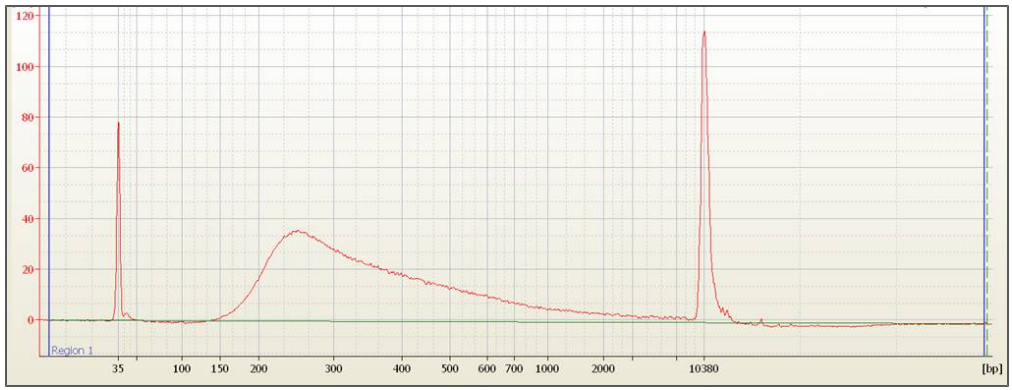
20 Remove the adhesive seal from the NLT plate.

21 Transfer 20 µl of clear supernatant from each well of the NLT plate to the corresponding well of the new HSP plate labeled NLA. Take care not to disturb the beads.

NOTE
Illumina recommends using a 20 µl single channel or multichannel pipette set to 10 µl to perform two consecutive transfers of 10 µl to reduce sample loss by ensuring that all of the liquid is transferred without disturbing the beads.

22 [Optional] Load 1 µl of the tagmentation reaction remaining in the NLT plate on an Agilent Technologies 2100 Bioanalyzer using a Agilent High Sensitivity DNA chip. Check the size of the sample, which should produce a broad distribution of DNA fragments with a size range from approximately 150 bp–1 kb.

Figure 2 Example TruSight Rapid Capture Post-Tagmentation Library Distribution



First PCR Amplification

This process amplifies the purified tagmented DNA via a -cycle PCR program. It also adds index 1 (i7) and index 2 (i5) needed for sequencing, as well as common adapters (P5 and P7) required for cluster generation and sequencing. It is critical to use the full amount of recommended input DNA and it is imperative that no extra cycles are added to the PCR process to ensure the generation of libraries that produce high-quality sequencing results.

Consumables

Item	Quantity	Storage	Supplied By
Index 1 primers (i7, N701–N712)	1 tube each index	-15° to -25°C	Illumina
Index 1 Tube Caps, Orange	1 per Index 1 primer tube	15° to 30°C	Illumina
Index 2 primers (i5, E501–E502)	1 tube each index	-15° to -25°C	Illumina
Index 2 Tube Caps, White	1 per Index 2 primer tube	15° to 30°C	Illumina
Nextera Library Amplification Mix (NLM)	1 tube	-15° to -25°C	Illumina
1.7 ml microcentrifuge tubes	1 per index primer tube	15° to 30°C	User
Microseal 'A' film	1	15° to 30°C	User
Microseal 'B' adhesive seal	1	15° to 30°C	User
RNase/DNase-free Strip Tube and Caps (for multi-sample processing)	1	15° to 30°C	User
[Optional] TruSeq® Index Plate Fixture Kit	1	15° to 30°C	User

Preparation

- Remove the Nextera Library Amplification Mix from -15° to -25°C storage and thaw on ice.
- Remove the following from -15° to -25°C storage and thaw at room temperature:
  - Index 1 primers (i7, N701–N712) (only remove primers being used)
  - Index 2 primers (i5, E501–E502) (only remove primers being used)

NOTE
TruSight Rapid Capture kits are designed to work only with Index 2 primers with the "E" prefix. Index 2 primers from other sample prep kits should not be used.

- For multi-sample processing:
  - Use a multichannel pipette.
  - Dispense the Nextera Library Amplification Mix in equal volumes into each of the wells of an eight-tube strip.
- Pre-program the thermal cycler with the following program and save as **NLM AMP**:
  - Choose the pre-heat lid option and set to 100°C
  - 72°C for 3 minutes
  - 98°C for 30 seconds
  - 10 cycles of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 10°C

NOTE
Illumina has optimized the number of recommended PCR cycles for enrichment assays based upon the level of pre-enrichment sample pooling and the size of the oligonucleotide set. Do not add or reduce the cycles of PCR, as it may compromise data quality.

Setup Index Primers

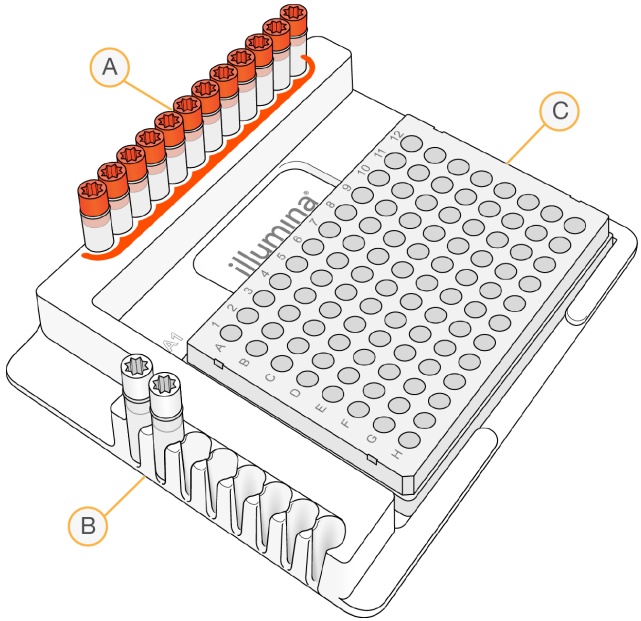
1. Vortex the index primer tubes for 5 seconds.
2. Centrifuge the index primer tubes to 600 xg for 5 seconds. Use empty 1.7 ml microcentrifuge tubes as tube adapters for the microcentrifuge.

3 Arrange the index primers in a rack on ice (i.e. the TruSeq Index Plate Fixture) using the following arrangement:

a Arrange the Index 1 Primer tubes (orange caps) vertically, aligned with columns 1-12.

b Arrange the Index 2 Primer tubes (white caps) horizontally, aligned with rows A-H.

Figure 3 Index Plate Fixture



A Index 1 Primer tubes (orange caps)
B Index 2 Primer tubes (white caps)
C NLA plate

Procedure

NOTE

For pooling libraries prior to enrichment, it is recommended to pool libraries so all Index 1 (i7) indices are unique. Choose Index 1 and Index 2 primers for PCR accordingly. For information, see *Low-Plex Pooling Guidelines* on page 65.

1. Add 5 µl of Index 1 (i7, N7xx) primer to each well of the NLA plate.
2. Add 5 µl of Index 2 (i5, E5xx) primer to each well of the NLA plate.
3. Add 20 µl of Nextera Library Amplification Mix to each well of the NLA plate.
4. Mix thoroughly as follows:
   a. Seal the NLA plate with a Microseal 'A' film.
   b. Shake the NLA plate on a microplate shaker at 1200 rpm for 1 minute
5. Centrifuge the NLA plate to 280 xg for 1 minute.
6. Place the sealed NLA plate on the pre-programmed thermal cycler. Close the lid, then select and run the **NLM AMP** program.

SAFE STOPPING POINT

If you do not plan to immediately proceed to *First PCR Clean Up* on page 18, the NLA plate can remain on the thermocycler overnight. If you are stopping, replace the Microseal 'A' with a Microseal 'B' adhesive seal and store the NLA plate at 2° to 8°C for up to two days.

First PCR Clean Up

This process uses Sample Purification Beads to purify the library DNA and remove unwanted products.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube	2° to 8°C	Illumina
96-well HSP plate	1	15° to 30°C	User
96-well MIDI plate	1	15° to 30°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15° to 30°C	User
Microseal 'B' adhesive seals	3	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (for multi-sample processing)	3	15° to 30°C	User

Preparation

- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 4 for information on how to access TruSight Rapid Capture Best Practices on the Illumina website.
- Make sure that the Resuspension Buffer and Sample Purification Beads are at room temperature.
- For multi-sample processing:
  - Use a multichannel pipette.
  - Pour the Resuspension Buffer, Sample Purification Beads, and 80% EtOH into separate multichannel reagent reservoirs.
- Label a new 96-well MIDI plate **NLC** (Nextera Library Clean Up) with a smudge resistant pen.

- Label a new 96-well HSP plate **NLS** (Nextera Library Sample) with a smudge resistant pen.

Procedure

1. Remove the NLA plate from the thermocycler and centrifuge to 280 xg for 1 minute.
2. Remove the adhesive seal from the NLA plate.
3. Transfer 50 µl of clear supernatant from each well of the NLA plate to the corresponding well of the new 96-well MIDI plate labeled NLC.
4. Vortex the Sample Purification Beads until the beads are well dispersed, then add 90 µl of well-resuspended Sample Purification Beads to each well of the NLC plate.
5. Mix thoroughly as follows:
   a. Seal the NLC plate with a Microseal 'B' adhesive seal.
   b. Shake the NLC plate on a microplate shaker at 1800 rpm for 1 minute.
6. Incubate the NLC plate at room temperature for 10 minutes.
7. Centrifuge the NLC plate to 280 xg for 1 minute.
8. Remove the adhesive seal from the NLC plate.
9. Place the NLC plate on the magnetic stand for 2 minutes or until the liquid appears clear.
10. Carefully remove and discard all of the supernatant from each well of the NLC plate.

NOTE
Leave the NLC plate on the magnetic stand while performing the following 80% EtOH wash steps (11–13).

11. With the NLC plate remaining on the magnetic stand, slowly add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads. Wait 30 seconds.
12. Remove and discard the 80% EtOH from each well of the NLC plate.
13. Repeat steps 11 and 12 once for a total of two 80% EtOH washes.
14. Using a 20 µl single channel or multichannel pipette, remove any remaining 80% EtOH from each well of the NLC plate without disturbing the beads.
15. Let the NLC plate stand at room temperature for 10 minutes to dry on the magnetic stand.

16 Remove the NLC plate from the magnetic stand.

17 Add 27 µl of Resuspension Buffer to each well of the NLC plate. Do not touch the beads with the pipette tips.

18 Mix thoroughly as follows:
   a Seal the NLC plate with a Microseal 'B' adhesive seal.
   b Shake the NLC plate on a microplate shaker at 1800 rpm for 1 minute.

19 Incubate the NLC plate at room temperature for 2 minutes.

20 Centrifuge the NLC plate to 280 xg for 1 minute.

21 Remove the adhesive seal from the NLC plate.

22 Place the NLC plate on the magnetic stand for 2 minutes or until the liquid appears clear.

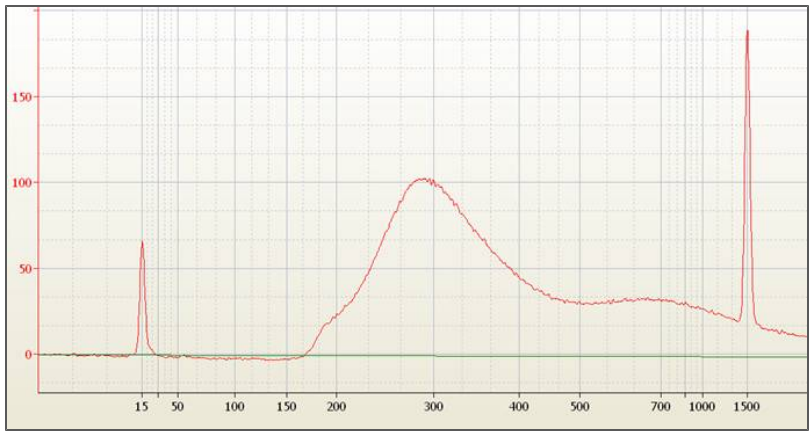
23 Transfer 25 µl of clear supernatant from each well of the NLC plate to the corresponding well of the new HSP plate labeled NLS. Take care not to disturb the beads.

NOTE
Illumina recommends using a 20 µl single channel or multichannel pipette set to 12.5 µl to perform two consecutive transfers of 12.5 µl to reduce sample loss by ensuring that all of the liquid is transferred without disturbing the beads.

24 Quantify the library in the NLS plate using a fluorometric method. See *DNA Quantitation* on page 68 or an example protocol using the Promega QuantiFluor method.

25 [Optional] Load 1 µl of the library on an Agilent Technologies 2100 Bioanalyzer using a Agilent DNA 1000 Chip. Check the size of the library, which should produce a distribution of DNA fragments with a size range from approximately 150 bp—1 kb.

Figure 4 Example TruSight Rapid Capture Post-PCR, Pre-Enriched Library Distribution



NOTE

The sample peak should not be significantly shifted compared to the example shown in Figure 4, although traces may differ depending on sample quality. A larger peak distribution (> 350 bp) can be indicative of > 50 ng gDNA input going into tagmentation and may result in lower on-target reads. Conversely, a smaller sample peak distribution (< 225 bp) can be indicative of < 50 ng gDNA or low quality gDNA, which may result in reduced library diversity or elevated duplicates.

SAFE STOPPING POINT

If you do not plan to proceed to *First Hybridization* on page 22 immediately, the protocol can be safely stopped here. If you are stopping, seal the NLS plate with a Microseal 'B' adhesive seal and store it at -15° to -25°C for up to 14 days.

First Hybridization

This process mixes the DNA library with capture probes to targeted regions of interest. The recommended hybridization time makes sure that targeted regions bind to the capture probes thoroughly. This process also describes how to combine multiple libraries with different indices into a single pool prior to enrichment.

Consumables

Item	Quantity	Storage	Supplied By
TruSight Content Set CSO (Custom Selected Oligos)	1 tube	-15° to -25°C	Illumina
Enrichment Hybridization Buffer (EHB)	1 tube	-15° to -25°C	Illumina
96-well HSP plate	1	15° to 30°C	User
[Optional] Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa)	1 per pooled sample	15° to 30°C	User
Microseal 'B' adhesive seal	1	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (for multi-sample processing)	2	15° to 30°C	User

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - Enrichment Hybridization Buffer
  - Custom Selected Oligos
- For multi-sample processing:
  - Use a multichannel pipette.
  - Distribute the Custom Selected Oligos and Enrichment Hybridization Buffer into separate eight-tube strips, dispensing equal volumes into each of the wells.
- Remove the NLS plate from -15° to -25°C storage, if it was stored at the conclusion of *First PCR Clean Up* and thaw on ice.

- Centrifuge the thawed NLS plate to 280 xg for 1 minute.
- Remove the adhesive seal from the thawed NLS plate.

▸ Pre-program the thermal cycler with the following program and save as **NRC HYB**:

a Choose the pre-heat lid option and set to 100°C
b 95°C for 10 minutes
c 18 cycles of 1 minute incubations, starting at 94°C, then decreasing 2°C per cycle
d 58°C for forever

▸ Label a new 96-well HSP plate **NEH1** (Nextera Enrichment Hyb 1) with a smudge resistant pen.

Pool Libraries

1 Reference the table below for the amount of DNA libraries to use for enrichment. Illumina recommends using 500 ng of each DNA library, quantified by QuantiFluor. See *DNA Input Recommendations* on page 3.

- If you are pooling libraries, combine 500 ng of each DNA library.
- If the total volume is greater than 40 µl, concentrate the pooled sample using either a vacuum concentrator or Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) according to manufacturer's instructions.
  - If you are using a vacuum concentrator, Illumina recommends concentrating samples with a no heat and medium drying rate setting.
  - If you are using a Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa), it is not required to pre-rinse the device prior to use. The majority of volume will filter through in 5 minutes, but up to 30 minutes may be required depending on the starting volume.
- If the pooled sample volume after concentrating is less than 40 µl, bring the volume up to 40 µl with Resuspension Buffer.

2 The recommended pre-enrichment pooling strategy is to pool libraries so that each contains a unique Index 1/i7 index. With this pooling approach samples can be sequenced using a single index read workflow, as described in the HiSeq® and GAIIx user guides.

- If Index1/i7 indexes are not unique, ensure that libraries with different Index 2/i5 indexes are included (e.g. N703/E501 and N703/E502). With this approach, sequence samples using a dual index read workflow, as described in the HiSeq and GAIIx user guides.
- See *Low-Plex Pooling Guidelines* on page 65 for further details.

Table 1 DNA Libraries for Enrichment

Library Pool Complexity	Total DNA Library Mass (ng)
1-plex	500
2-plex	1000
3-plex	1500
4-plex	2000
5-plex	2500
6-plex	3000
7-plex	3500
8-plex	4000
9-plex	4500
10-plex	5000
11-plex	5500
12-plex	6000

Procedure

1 Thoroughly vortex the Enrichment Hybridization Buffer tube until the solution is completely resuspended. Visually make sure that no crystal structures are present.

NOTE
If crystals and cloudiness are observed, vortex the Enrichment Hybridization Buffer tube until it appears clear.

2 Add the following reagents in the order listed below to each well of the new 96-well HSP plate labeled NEH1.

Reagent	Volume (µl)
DNA library sample or library pool from NLS plate	40
Enrichment Hybridization Buffer	50
TruSight Content Set CSO	10
Total Volume per Sample	**100**

3 Mix thoroughly as follows:
   a Seal the NEH1 plate with a Microseal 'B' adhesive seal. Make sure that the plate is tightly sealed to prevent potential evaporation. Use an adhesive seal roller to apply force to the seal and make sure the seal is secured.
   b Shake the NEH1 plate on a microplate shaker at 1200 rpm for 1 minute.

4 Centrifuge the NEH1 plate to 280 xg for 1 minute.

5 Place the sealed NEH1 plate on the pre-programmed thermal cycler. Close the lid, then select and run the **NRC HYB** program.
Incubate the plate at the 58°C holding temperature for at least 90 minutes and up to a maximum of 24 hours. Do not remove the plate from 58°C incubation until you are ready to proceed to *First Capture* on page 26.

First Capture

This process uses streptavidin beads to capture probes hybridized to the targeted regions of interest. Two heated wash procedures remove non-specific binding from the beads. The enriched library is then eluted from the beads and prepared for a second round of hybridization.

Consumables

Item	Quantity	Storage	Supplied By
2N NaOH (HP3)	1 tube	-15° to -25°C	Illumina
Elute Target Buffer 2 (ET2)	1 tube	2° to 8°C	Illumina
Enrichment Elution Buffer 1 (EE1)	1 tube	-15° to -25°C	Illumina
Enrichment Wash Solution (EWS)	1 tube	-15° to -25°C	Illumina
Streptavidin Magnetic Beads (SMB)	1 tube	2° to 8°C	Illumina
1.7 ml microcentrifuge tube	1	15° to 30°C	User
96-well HSP plate	1	15° to 30°C	User
96-well MIDI plate	1	15° to 30°C	User
Microseal 'B' adhesive seals	6	15° to 30°C	User

Preparation

- Remove the 2N NaOH, Enrichment Elution Buffer 1, and Enrichment Wash Solution from -15° to -25°C storage and thaw at room temperature.
- Remove the Elute Target Buffer 2 and Streptavidin Magnetic Beads from 2° to 8°C storage and let stand at room temperature.

  NOTE
  Make sure that you use the Streptavidin Magnetic Beads (2 ml tube) and not the Sample Purification Beads (15 ml tube) for this procedure.

- Pre-heat the microheating system to 50°C.

- Label a new 96-well MIDI plate **NEW1** (Nextera Enrichment Wash 1) with a smudge resistant pen.
- Label a new 96-well HSP plate **NEH2** (Nextera Enrichment Hyb 2) with a smudge resistant pen.

First Bind

1. Remove the NEH1 plate from the thermal cycler.
2. Centrifuge the NEH1 plate to 280 xg for 1 minute.
3. Remove the adhesive seal from the NEH1 plate. Take care when removing the seal to avoid spilling the contents of the wells.
4. Transfer the entire contents (~100 µl) from each well of the NEH1 plate to the corresponding well of the new 96-well MIDI plate labeled NEW1.

   NOTE

   If an overnight First Hybridization was performed, it is normal to see a small degree of sample loss. However, if the sample loss is greater than 15%, Illumina does not recommended proceeding with the sample preparation. This amount of loss can be caused by poor sealing or not heating the lid.

5. Vortex the Streptavidin Magnetic Beads tube until the beads are well dispersed, then add 250 µl of well-mixed Streptavidin Magnetic Beads to the wells of the NEW1 plate.
6. Mix thoroughly as follows:
   a. Seal the NEW1 plate with a Microseal 'B' adhesive seal.
   b. Shake the NEW1 plate on a microplate shaker at 1200 rpm for 5 minutes.
7. Let the NEW1 plate stand at room temperature for 25 minutes.
8. Centrifuge the NEW1 plate to 280 xg for 1 minute.
9. Remove the adhesive seal from the NEW1 plate.
10. Place the NEW1 plate on the magnetic stand for 2 minutes at room temperature or until the liquid appears clear.
11. Carefully remove and discard all of the supernatant from each well of the NEW1 plate without disturbing the beads.
12. Remove the NEW1 plate from the magnetic stand.

First Wash

1 Make sure that the Enrichment Wash Solution tube is at room temperature, then thoroughly vortex the tube.

NOTE
It is normal that the Enrichment Wash Solution may be cloudy after vortexing.

2 Add 200 µl of Enrichment Wash Solution to each well of the NEW1 plate.

3 Mix thoroughly as follows:
   a Seal the NEW1 plate with a Microseal 'B' adhesive seal.
   b Shake the NEW1 plate on a microplate shaker at 1800 rpm for 4 minutes.
   c Remove the adhesive seal from the NEW1 plate.
   d Gently pipette the entire volume of each well up and down to ensure complete resuspension of the sample.

4 Seal the NEW1 plate with a Microseal 'B' adhesive seal.

5 Place the sealed NEW1 plate on the **pre-heated** microheating system. Close the lid and incubate at 50°C for 30 minutes.

6 Place the magnetic stand next to the microheating system for immediate access.

7 Remove the NEW1 plate from the microheating system and ***immediately*** place it on the magnetic stand for 2 minutes or until the liquid appears clear.

8 Remove the adhesive seal from the NEW1 plate.

9 Immediately remove and discard all of the supernatant from each well of the NEW1 plate.

10 Remove the NEW1 plate from the magnetic stand.

11 Repeat steps 2–10 once for a total of two Enrichment Wash Solution washes.

First Elution

1 Add the following reagents in the order listed in a new 1.7 ml microcentrifuge tube to create the elution pre-mix. Multiply each volume by the number of pooled samples being prepared. The volumes below include an excess amount for processing multiple samples.

Reagent	Volume (µl)
Enrichment Elution Buffer 1	28.5
2N NaOH	1.5
Total Volume per Sample	**30**

2 Vortex the elution pre-mix tube, then add **23 µl** of the mix to each well of the NEW1 plate.

3 Mix thoroughly as follows:
   a Seal the NEW1 plate with a Microseal 'B' adhesive seal.
   b Shake the NEW1 plate on a microplate shaker at 1800 rpm for 2 minutes.

4 Let the NEW1 plate stand at room temperature for 2 minutes.

5 Centrifuge the NEW1 plate to 280 xg for 1 minute.

6 Carefully remove the adhesive seal from the NEW1 plate to avoid spilling the contents of the wells.

7 Place the NEW1 plate on the magnetic stand for 2 minutes or until the liquid appears clear.

8 Transfer 21 µl of clear supernatant from each well of the NEW1 plate to the corresponding well of the new HSP plate labeled NEH2. Take care not to disturb the beads.

NOTE
Illumina recommends using a 20 µl single channel or multichannel pipette set to 10.5 µl to perform two consecutive transfers of 10.5 µl to reduce sample loss by ensuring that all of the liquid is transferred without disturbing the beads.

9 Add 4 µl of Elute Target Buffer 2 to each well of the NEH2 plate containing samples to neutralize the elution.

10 Mix thoroughly as follows:
   a Seal the NEH2 plate with a Microseal 'B' adhesive seal.
   b Shake the NEH2 plate on a microplate shaker at 1200 rpm for 1 minute.

11 Centrifuge the NEH2 plate to 280 xg for 1 minute.

12 Store the remaining reagents as follows:
   a Place the Elute Target Buffer 2 and Streptavidin Magnetic Beads tubes in 2° to 8°C storage.
   b Place the 2N NaOH, Enrichment Elution Buffer 1, and Enrichment Wash Solution tubes in -15° to -25°C storage.
   c Discard any remaining elution pre-mix.

SAFE STOPPING POINT

If you do not plan to proceed to *Second Hybridization* on page 31 immediately, the protocol can be safely stopped here. If you are stopping, seal the NEH2 plate with a Microseal 'B' adhesive seal and store it at -15° to -25°C for up to seven days.

Second Hybridization

This process combines the eluted DNA library from the first enrichment round with additional capture probes to targeted regions of interest. This second hybridization is required to ensure high specificity of the captured regions.

Consumables

Item	Quantity	Storage	Supplied By
TruSight Content Set CSO (Custom Selected Oligos)	1 tube	-15° to -25°C	Illumina
Enrichment Hybridization Buffer (EHB)	1 tube	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
Microseal 'B' adhesive seal	1	15° to 30°C	User

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - Enrichment Hybridization Buffer
  - Custom Selected Oligos
- Make sure that the Resuspension Buffer is at room temperature.
- Remove the NEH2 plate from -15° to -25°C storage, if it was stored at the conclusion of *First Capture* and thaw on ice.
  - Centrifuge the thawed NEH2 plate to 280 xg for 1 minute.

Procedure

1 Thoroughly vortex the Enrichment Hybridization Buffer tube until the solution is completely resuspended. Visually make sure that no crystal structures are present.

NOTE
If crystals and cloudiness are observed, vortex the Enrichment Hybridization Buffer tube until it appears clear.

2 Remove the adhesive seal from the NEH2 plate.

3 Add 15 µl of Resuspension Buffer to each well of the NEH2 plate.

4 Add 50 µl of Enrichment Hybridization Buffer to each well of the NEH2 plate.

5 Add 10 µl of the CSO tube to each well of the NEH2 plate.

6 Mix thoroughly as follows:
   a Seal the NEH2 plate with a Microseal 'B' adhesive seal. Make sure that the plate is tightly sealed to prevent potential evaporation. Use an adhesive seal roller to apply force to the seal and make sure the seal is secured.
   b Shake the NEH2 plate on a microplate shaker at 1200 rpm for 1 minute

7 Centrifuge the NEH2 plate to 280 xg for 1 minute.

8 Place the sealed NEH2 plate on the pre-programmed thermal cycler. Close the lid, then select and run the **NRC HYB** program.
Incubate the plate at the 58°C holding temperature overnight for at least 14.5 hours and up to a maximum of 24 hours. Do not remove the plate from 58°C incubation until you are ready to proceed to *Second Capture* on page 33.

Second Capture

This process uses streptavidin beads to capture probes hybridized to the targeted regions of interest. Two heated wash procedures remove non-specific binding from the beads. The enriched library is then eluted from the beads and prepared for sequencing.

NOTE
These procedures are similar to the *First Capture* on page 26.

Consumables

Item	Quantity	Storage	Supplied By
2N NaOH (HP3)	1 tube	-15° to -25°C	Illumina
Elute Target Buffer 2 (ET2)	1 tube	2° to 8°C	Illumina
Enrichment Elution Buffer 1 (EE1)	1 tube	-15° to -25°C	Illumina
Enrichment Wash Solution (EWS)	1 tube	-15° to -25°C	Illumina
Streptavidin Magnetic Beads (SMB)	1 tube	2° to 8°C	Illumina
1.7 ml microcentrifuge tube	1	15° to 30°C	User
96-well MIDI plates	2	15° to 30°C	User
Microseal 'B' adhesive seals	6	15° to 30°C	User

Preparation

- Remove the 2N NaOH, Enrichment Elution Buffer 1, and Enrichment Wash Solution from -15° to -25°C storage and thaw at room temperature.
- Remove the Elute Target Buffer 2 and Streptavidin Magnetic Beads from 2° to 8°C storage and let stand at room temperature.

NOTE
Make sure that you use the Streptavidin Magnetic Beads (2 ml tube) and not the Sample Purification Beads (15 ml tube) for this procedure.

- Pre-heat the microheating system to 50°C.

- Label a new 96-well MIDI plate **NEW2** (Nextera Enrichment Wash 2) with a smudge resistant pen.
- Label a new 96-well MIDI plate **NEC1** (Nextera Enriched Clean Up 1) with a smudge resistant pen.

Second Bind

1. Remove the NEH2 plate from the thermal cycler.
2. Centrifuge the room temperature NEH2 plate to 280 xg for 1 minute.
3. Remove the adhesive seal from the NEH2 plate. Take care when removing the seal to avoid spilling the contents of the wells.
4. Transfer the entire contents (~100 µl) from each well of the NEH2 plate to the corresponding well of the new 96-well MIDI plate labeled NEW2.

   NOTE
   It is normal to see a small degree of sample loss after overnight hybridization. However, if the sample loss is greater than 15%, Illumina does not recommended proceeding with the sample preparation. This amount of loss can be caused by poor sealing or not heating the lid.

5. Vortex the Streptavidin Magnetic Beads tube until the beads are well dispersed, then add 250 µl of well-mixed Streptavidin Magnetic Beads to the wells of the NEW2 plate.
6. Mix thoroughly as follows:
   a. Seal the NEW2 plate with a Microseal 'B' adhesive seal.
   b. Shake the NEW2 plate on a microplate shaker at 1200 rpm for 5 minutes
7. Let the NEW2 plate stand at room temperature for 25 minutes.
8. Centrifuge the NEW2 plate to 280 xg for 1 minute.
9. Remove the adhesive seal from the NEW2 plate.
10. Place the NEW2 plate on the magnetic stand for 2 minutes at room temperature or until the liquid appears clear.
11. Carefully remove and discard all of the supernatant from each well of the NEW2 plate without disturbing the beads.
12. Remove the NEW2 plate from the magnetic stand.

Second Wash

1 Make sure that the Enrichment Wash Solution tube is at room temperature, then thoroughly vortex the tube.

NOTE
It is normal that the Enrichment Wash Solution may be cloudy after vortexing.

2 Add 200 µl of Enrichment Wash Solution to each well of the NEW2 plate.

3 Mix thoroughly as follows:
   a Seal the NEW2 plate with a Microseal 'B' adhesive seal.
   b Shake the NEW2 plate on a microplate shaker at 1800 rpm for 4 minutes
   c Remove the adhesive seal from the NEW2 plate.
   d Gently pipette the entire volume of each well up and down to ensure complete resuspension of the sample.

4 Seal the NEW2 plate with a Microseal 'B' adhesive seal.

5 Incubate the NEW2 plate on the **pre-heated** microheating system, with the lid closed, at 50°C for 30 minutes.

6 Place the magnetic stand next to the microheating system for immediate access.

7 Remove the NEW2 plate from the microheating system and ***immediately*** place it on the magnetic stand for 2 minutes or until the liquid appears clear.

8 Remove the adhesive seal from the NEW2 plate.

9 Immediately remove and discard all of the supernatant from each well of the NEW2 plate.

10 Remove the NEW2 plate from the magnetic stand.

11 Repeat steps 2–10 once for a total of two Enrichment Wash Solution washes.

Second Elution

1 Add the following reagents in the order listed in a new 1.7 ml microcentrifuge tube to create the elution pre-mix. Multiply each volume by the number of pooled samples being prepared. The volumes below include an excess amount for processing multiple samples.

Reagent	Volume (µl)
Enrichment Elution Buffer 1	28.5
2N NaOH	1.5
Total Volume per Sample	**30**

2 Vortex the elution pre-mix tube, then add **23 µl** of the mix to each well of the NEW2 plate.

3 Mix thoroughly as follows:
   a Seal the NEW2 plate with a Microseal 'B' adhesive seal.
   b Shake the NEW2 plate on a microplate shaker at 1800 rpm for 2 minutes.

4 Let the NEW2 plate stand at room temperature for 2 minutes.

5 Centrifuge the NEW2 plate to 280 xg for 1 minute.

6 Carefully remove the adhesive seal from the NEW2 plate to avoid spilling the contents of the wells.

7 Place the NEW2 plate on the magnetic stand for 2 minutes or until the liquid appears clear.

8 Transfer 21 µl of clear supernatant from each well of the NEW2 plate to the corresponding well of the new MIDI plate labeled NEC1. Take care not to disturb the beads.

NOTE
Illumina recommends using a 20 µl single channel or multichannel pipette set to 10.5 µl to perform two consecutive transfers of 10.5 µl to reduce sample loss by ensuring that all of the liquid is transferred without disturbing the beads.

9 Add 4 µl Elute Target Buffer 2 to each well of the NEC1 plate containing samples to neutralize the elution.

10 Mix thoroughly as follows:
   a Seal the NEC1 plate with a Microseal 'B' adhesive seal.
   b Shake the NEC1 plate on a microplate shaker at 1800 rpm for 1 minute.

11 Centrifuge the NEC1 plate to 280 xg for 1 minute.

12 Store the remaining reagents as follows:
   a Place the Elute Target Buffer 2 and Streptavidin Magnetic Beads tubes in 2° to 8°C storage.
   b Place the 2N NaOH, Enrichment Elution Buffer 1, and Enrichment Wash Solution tubes in -15° to -25°C storage.
   c Discard any remaining elution pre-mix.

Capture Sample Clean Up

This process uses Sample Purification Beads to purify the captured library prior to PCR amplification.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube	2° to 8°C	Illumina
96-well HSP plate	1	15° to 30°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15° to 30°C	User
Microseal 'B' adhesive seals	3	15° to 30°C	User

Preparation

- Make sure that the Resuspension Buffer and Sample Purification Beads are at room temperature.
- Label a new 96-well HSP plate **NEA** (Nextera Enrichment Amplification) with a smudge resistant pen.

Procedure

1. Remove the adhesive seal from the NEC1 plate.
2. Vortex the Sample Purification Beads tube until the beads are well dispersed, then add 45 µl of the well-mixed Sample Purification Beads to each well of the NEC1 plate.
3. Mix thoroughly as follows:
   a. Seal the NEC1 plate with a Microseal 'B' adhesive seal.
   b. Shake the NEC1 plate on a microplate shaker at 1800 rpm for 1 minute.
4. Incubate the NEC1 plate at room temperature for 10 minutes.
5. Centrifuge the NEC1 plate to 280 xg for 1 minute.

6 Remove the adhesive seal from the NEC1 plate.

7 Place the NEC1 plate on the magnetic stand for 2 minutes or until the liquid appears clear.

8 Remove and discard all of the supernatant from each well of the NEC1 plate.

NOTE
Leave the NEC1 plate on the magnetic stand while performing the following 80% EtOH wash steps (9–11).

9 With the NEC1 plate remaining on the magnetic stand, slowly add 200 µl of freshly made 80% EtOH to each well without disturbing the beads. Wait 30 seconds.

10 Remove and discard the 80% EtOH from each well of the NEC1 plate.

11 Repeat steps 9 and 10 once for a total of two 80% EtOH washes.

12 Using a 20 µl single channel or multichannel pipette, remove any remaining 80% EtOH from each well of the NEC1 plate without disturbing the beads.

13 Let the NEC1 plate stand at room temperature for 10 minutes to dry on the magnetic stand.

14 Remove the NEC1 plate from the magnetic stand.

15 Add 27.5 µl of Resuspension Buffer to each well of the NEC1 plate. Do not touch the beads with the pipette tips.

16 Mix thoroughly as follows:
   a Seal the NEC1 plate with a Microseal 'B' adhesive seal.
   b Shake the NEC1 plate on a microplate shaker at 1800 rpm for 1 minute.

17 Incubate the NEC1 plate at room temperature for 2 minutes.

18 Centrifuge the NEC1 plate to 280 xg for 1 minute.

19 Remove the adhesive seal from the NEC1 plate.

20 Place the NEC1 plate on the magnetic stand for 2 minutes or until the liquid appears clear.

21 Transfer 25 µl of clear supernatant from each well of the NEC1 plate to the corresponding well of the new HSP plate labeled NEA. Take care not to disturb the beads.

NOTE

Illumina recommends using a 20 µl single channel or multichannel pipette set to 12.5 µl to perform two consecutive transfers of 12.5 µl to reduce sample loss by ensuring that all of the liquid is transferred without disturbing the beads.

SAFE STOPPING POINT

If you do not plan to proceed to *Second PCR Amplification* on page 41 immediately, the protocol can be safely stopped here. If you are stopping, seal the NEA plate with a Microseal 'B' adhesive seal and store it at -15° to -25°C for up to seven days.

Second PCR Amplification

This process amplifies the captured library via a 12-cycle PCR program. It is critical to use the full amount of recommended input DNA and not add extra PCR cycles to ensure libraries produce high-quality sequencing results.

Consumables

Item	Quantity	Storage	Supplied By
Nextera Enrichment Amplification Mix (NEM)	1 tube	-15° to -25°C	Illumina
PCR Primer Cocktail (PPC)	1 tube	-15° to -25°C	Illumina
Microseal 'A' film	1	15° to 30°C	User
Microseal 'B' adhesive seal	1	15° to 30°C	User

Preparation

- Remove the Nextera Enrichment Amplification Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw on ice.
  - Briefly centrifuge the thawed Nextera Enrichment Amplification Mix and PCR Primer Cocktail tubes for 5 seconds.

NOTE
If you do not intend to consume the Nextera Enrichment Amplification Mix and PCR Primer Cocktail in one use, dispense the reagents into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- Remove the NEA plate from -15° to -25°C storage, if it was stored at the conclusion of *Second Capture* and thaw on ice.
  - Centrifuge the thawed NEA plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed NEA plate.

- Pre-program the thermal cycler with the following program and save as **NEM AMP**:
  - Choose the pre-heat lid option and set to 100°C
  - 98°C for 30 seconds
  - 12 cycles of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 10°C

NOTE
Illumina has optimized the number of recommended PCR cycles for enrichment assays based upon the level of pre-enrichment sample pooling and the size of the oligonucleotide set. Do not add or reduce the cycles of PCR, as it may compromise data quality.

Procedure

1. Add 5 µl of PCR Primer Cocktail to each well of the NEA plate.
2. Add 20 µl of Nextera Enrichment Amplification Mix to each well of the NEA plate.
3. Mix thoroughly as follows:
   a. Seal the NEA plate with a Microseal 'A' film. Use an adhesive seal roller to apply force to the film and make sure the film is secured.
   b. Shake the NEA plate on a microplate shaker at 1200 rpm for 1 minute
4. Centrifuge the NEA plate to 280 xg for 1 minute.
5. Place the sealed NEA plate on the pre-programmed thermal cycler. Close the lid, then select and run the **NEM AMP** program.

SAFE STOPPING POINT
If you do not plan to immediately proceed to *Second PCR Clean Up* on page 43, the NEA plate can remain on the thermocycler overnight. If you are stopping, replace the Microseal 'A' with a Microseal 'B' adhesive seal and store the NEA plate at 2° to 8°C for up to two days.

Second PCR Clean Up

This process uses Sample Purification Beads to purify the enriched library and remove unwanted products.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube	2° to 8°C	Illumina
96-well HSP plate	1	15° to 30°C	User
96-well MIDI plate	1	15° to 30°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15° to 30°C	User
Microseal 'B' adhesive seals	3	15° to 30°C	User

Preparation

- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 4 for information on how to access TruSight Rapid Capture Best Practices on the Illumina website.
- Make sure that the Resuspension Buffer and Sample Purification Beads are at room temperature.
- Remove the NEA plate from 2° to 8°C storage, if it was stored at the conclusion of *Second PCR Amplification* and let stand to bring to room temperature.
- Label a new 96-well MIDI plate **NEC2** (Nextera Enriched Clean Up 2) with a smudge resistant pen.
- Label a new 96-well HSP plate **NES** (Nextera Enrichment Sample) with a smudge resistant pen.

Procedure

1. Centrifuge the NEA plate to 280 xg for 1 minute.
2. Remove the adhesive seal from the NEA plate.
3. Transfer the entire contents from each well of the NEA plate to the corresponding well of the new 96-well MIDI plate labeled NEC2.
4. Vortex the Sample Purification Beads until the beads are well dispersed, then add 90 µl of the well-mixed Sample Purification Beads to each well of the NEC2 plate containing 50 µl of the PCR amplified library.
5. Mix thoroughly as follows:
   a. Seal the NEC2 plate with a Microseal 'B' adhesive seal.
   b. Shake the NEC2 plate on a microplate shaker at 1800 rpm for 1 minute.
6. Incubate the NEC2 plate at room temperature for 10 minutes.
7. Centrifuge the NEC2 plate to 280 xg for 1 minute.
8. Remove the adhesive seal from the NEC2 plate.
9. Place the NEC2 plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.
10. Carefully remove and discard all of the supernatant from each well of the NEC2 plate.

NOTE
Leave the NEC2 plate on the magnetic stand while performing the following 80% EtOH wash steps (11–13).

11. With the NEC2 plate remaining on the magnetic stand, slowly add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads. Wait 30 seconds.
12. Remove and discard the 80% EtOH from each well of the NEC2 plate.
13. Repeat steps 11–12 once for a total of two 80% EtOH washes.
14. Using a 20 µl single channel or multichannel pipette, remove any remaining 80% EtOH from each well of the NEC2 plate without disturbing the beads.
15. Let the NEC2 plate stand at room temperature for 10 minutes to dry on the magnetic stand.
16. Remove the NEC2 plate from the magnetic stand.

17 Add 32 µl of Resuspension Buffer to each well of the NEC2 plate. Do not touch the beads with the pipette tips.

18 Mix thoroughly as follows:
   a Seal the NEC2 plate with a Microseal 'B' adhesive seal.
   b Shake the NEC2 plate on a microplate shaker at 1800 rpm for 1 minute.

19 Incubate the NEC2 plate at room temperature for 2 minutes.

20 Centrifuge the NEC2 plate to 280 xg for 1 minute.

21 Remove the adhesive seal from the NEC2 plate.

22 Place the NEC2 plate on the magnetic stand for 2 minutes or until the liquid appears clear.

23 Transfer 30 µl of clear supernatant from each well of the NEC2 plate to the corresponding well of the new HSP plate labeled NES. Take care not to disturb the beads.

NOTE

Illumina recommends using a 20 µl single channel or multichannel pipette set to 15 µl to perform two consecutive transfers of 15 µl to reduce sample loss by ensuring that all of the liquid is transferred without disturbing the beads.

24 Seal the NES plate with a Microseal 'B' adhesive seal.

SAFE STOPPING POINT

If you do not plan to immediately proceed to *Validate Library* on page 46, store the sealed NES plate at -15° to -25°C for up to seven days. If the plate is stored for more than seven days, re-quantify your library to guarantee the accuracy of your enrichment results.

Validate Library

Illumina recommends performing the following procedures for quality control analysis and quantification of your enriched library.

Quantify Libraries

In order to achieve the highest data quality on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of every flow cell. This requires accurate quantitation of DNA library templates.

Quantify the post-enriched library in the NES plate using a fluorometric method. See *DNA Quantitation* on page 68 for an example protocol using the Promega QuantiFluor method.

Alternatively, you can quantitate libraries using qPCR according to the *Sequencing Library qPCR Quantification Guide (part # 11322363)*.

NOTE

You can download the *Sequencing Library qPCR Quantification Guide* from the Illumina website at support.illumina.com/sequencing/kits.ilmn.

Click **TruSight Rapid Capture Sample Prep Kit Support**. Then click **Documentation & Literature** on **TruSight Rapid Capture Sample Prep Kit Support**.

NOTE

Use the following formula to convert from ng/µl to nM. Assume a 400 bp library size or calculate based on the average size of the enriched library:

$$\frac{\text{(concentration in ng/µl)}}{\text{(660 g/mol *average library size)}} \times 10^6 = \text{concentration in nM}$$

For example:

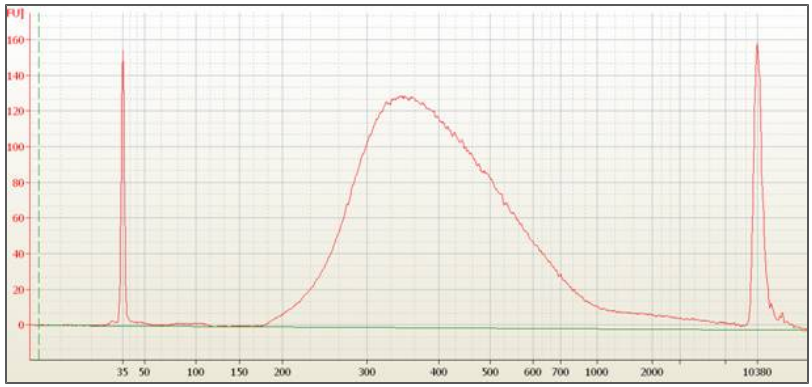
$$\frac{\text{15 ng/µl)}}{\text{(660 g/mol *400)}} \times 10^6 = 57 \text{ nM}$$

Assess Quality [Optional]

To assess library quality, load 1 µl of the post-enriched library on an Agilent Technologies 2100 Bioanalyzer using a Agilent DNA High Sensitivity Chip. Depending on the level of indexing, an initial dilution of your sample may be necessary. For a 12-plex pool, Illumina recommends a 1:10 dilution of your sample prior to loading.

Check the size of the library, which should produce a distribution of DNA fragments with a size range from approximately 200 bp—1 kb. Depending on the level of indexing, insert size distribution may vary slightly, however the sample peak should not be significantly shifted compared to the example in Figure 5.

Figure 5 Example TruSight Rapid Capture Post-Enrichment (12-plex Enrichment) Library Distribution



NOTE

A second minor peak at ~100 bp may be present and and likely corresponds to residual single-stranded probes in the sample. The presence of these residual probes will not impact downstream clustering and sequencing of your enriched sample.

Sequence Library

Proceed to cluster generation. For more information, see the cluster generation section of the user guide for your Illumina platform.

- When quantifying a TruSight Rapid Capture post-enriched library using a fluorometric method, clustering at 8–12 pM generates cluster densities in the range of 750,000—950,000 clusters/mm2. Results may vary based on your method of quantification. Illumina recommends that you determine the library concentration to cluster density relationship based on your lab instrumentation.
- A short sequencing run on a MiSeq® can also be performed to optimize cluster density prior to performing a high density sequencing run.
- TruSight Rapid Capture prepared libraries contain dual 8 bp indexes. Depending on the combination of indexes used in your library pool, configure the sequencing run for either single or dual 8 bp index reads. See the *Low-Plex Pooling Guidelines* on page 65 for additional information.
- When clustering TruSight Rapid Capture libraries on the cBot™ and sequencing on the HiSeq 1000/2000, GAIIx, or in High Output mode on the HiSeq 1500/2500, new primers are required whether performing a non-indexed, single-indexed, or dual-indexed run. Use the TruSeq Dual Index Sequencing Primer Kit for Paired End runs (catalog # PE-121-1003), which is good for a single run and contains the required primers for sequencing (HP10, HP11, HP12). These primers are included with MiSeq and HiSeq 1500/2500 rapid run reagent kits.
- For sequencing TruSight Rapid Capture libraries, Illumina recommends a paired-end 150-cycle sequencing run. Due to the library sizes generated in TruSight Rapid Capture, sequencing at longer read lengths can lead to an increase in the likelihood of sequencing into the flanking adapter sequence.

Supporting Information

Appendix A

Introduction

The protocols described in this guide assume that you are familiar with the contents of this appendix, have confirmed your kit contents, and have obtained all of the requisite equipment and consumables.

Acronyms

Table 2 TruSight Rapid Capture Acronyms

Acronym	Definition
dsDNA	Double-stranded DNA
EE1	Enrichment Elution Buffer 1
EHB	Enrichment Hybridization Buffer
ET2	Elute Target Buffer 2
EWS	Enrichment Wash Solution
gDNA	Genomic DNA
HP3	2N NaOH
HSP	Hard Shell Plate
NEA	Nextera Enrichment Amplification Plate
NEC1	Nextera Enriched Clean Up Plate 1
NEC2	Nextera Enriched Clean Up Plate 2
NEH1	Nextera Enrichment Hyb Plate 1
NEH2	Nextera Enrichment Hyb Plate 2
NEM	Nextera Enrichment Amplification Mix
NES	Nextera Enrichment Sample Plate
NEW1	Nextera Enrichment Wash Plate 1
NEW2	Nextera Enrichment Wash Plate 2
NLA	Nextera Library Amplification Plate
NLC	Nextera Library Clean Up Plate

Acronym	Definition
NLM	Nextera Library Amplification Mix
NLS	Nextera Library Sample Plate
NLT	Nextera Library Tagment Plate
PCR	Polymerase Chain Reaction
PPC	PCR Primer Cocktail
RCO	Rapid Capture Oligos
RFU	Relative Fluorescence Unit
RSB	Resuspension Buffer
SMB	Streptavidin Magnetic Beads
SPB	Sample Purification Beads
ST	Stop Tagment Buffer
TD	Tagment DNA Buffer
TDE1	Tagment DNA Enzyme 1

Kit Contents

Check to make sure that you have all of the reagents identified in this section before proceeding to the sample preparation procedures. TruSight Rapid Capture kits are available in the following configurations.

Table 3 TruSight Rapid Capture Kits

Kit Name	Catalog #	*TG Catalog #
TruSight Rapid Capture Kit (1 Index, 8 Samples)	FC-140-1101	TG-140-1101
TruSight Rapid Capture Kit (2 Indices, 8 Samples)	FC-140-1102	TG-140-1102
TruSight Rapid Capture Kit (4 Indices, 16 Samples)	FC-140-1103	TG-140-1103
TruSight Rapid Capture Kit (24 Indices, 48 Samples)	FC-140-1104	TG-140-1104
TruSight Rapid Capture Kit (24 Indices, 96 Samples)	FC-140-1105	TG-140-1105
TruSight Rapid Capture Kit (96 Indices, 288 Samples)	FC-140-1106	TG-140-1106

NOTE

*TG-labeled consumables include features intended to help reduce the frequency of revalidation. They are available only under supply agreement and require you to provide a binding forecast. Please contact your account manager for more information.

Note regarding biomarker patents and other patents unique to specific uses of products.

Some genomic variants, including some nucleic acid sequences, and their use in specific applications may be protected by patents. Customers are advised to determine whether they are required to obtain licenses from the party that owns or controls such patents in order to use the product in customer's specific application.

TruSight Rapid Capture Kit Contents (1 Index, 8 Samples) (FC-140-1101, TG-140-1101)

Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
1	SPB	Sample Purification Beads	2° to 8°C
4	SMB	Streptavidin Magnetic Beads	2° to 8°C
1	ET2	Elute Target Buffer 2	2° to 8°C
1	ST	Stop Tagment Buffer	15° to 30°C

Box 2

Quantity	Acronym	Reagent Name	Storage Temperature
2	TDE1	Tagment DNA Enzyme	-15° to -25°C
2	EE1	Enrichment Elution Buffer 1	-15° to -25°C
2	TD	Tagment DNA Buffer	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C
1	NLM	Nextera Library Amplification Mix	-15° to -25°C
2	EHB	Enrichment Hybridization Buffer	-15° to -25°C
2	EWS	Enrichment Wash Solution	-15° to -25°C
1	HP3	2N NaOH	-15° to -25°C
1	PPC	PCR Primer Cocktail	-15° to -25°C
4	NEM	Nextera Enrichment Amplification Mix	-15° to -25°C

Box 3

Quantity	Reagent Name	Storage Temperature
1 tube	Index Primer, E501	-15° to -25°C
1 tube	Index Primer, N701	-15° to -25°C

TruSight Rapid Capture Kit Contents (2 Indices, 8 Samples) (FC-140-1102, TG-140-1102)

Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
1	SPB	Sample Purification Beads	2° to 8°C
2	SMB	Streptavidin Magnetic Beads	2° to 8°C
1	ET2	Elute Target Buffer 2	2° to 8°C
1	ST	Stop Tagment Buffer	15° to 30°C

Box 2

Quantity	Acronym	Reagent Name	Storage Temperature
1	TDE1	Tagment DNA Enzyme	-15° to -25°C
1	EE1	Enrichment Elution Buffer 1	-15° to -25°C
1	TD	Tagment DNA Buffer	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C
1	NLM	Nextera Library Amplification Mix	-15° to -25°C
1	EHB	Enrichment Hybridization Buffer	-15° to -25°C
1	EWS	Enrichment Wash Solution	-15° to -25°C
1	HP3	2N NaOH	-15° to -25°C
1	PPC	PCR Primer Cocktail	-15° to -25°C
2	NEM	Nextera Enrichment Amplification Mix	-15° to -25°C

Box 3

Quantity	Reagent Name	Storage Temperature
1 tube	Index Primer, E501	-15° to -25°C
2 tubes	Index Primer, N701 to N702	-15° to -25°C

TruSight Rapid Capture Kit Contents (4 Indices, 16 Samples) (FC-140-1103, TG-140-1103)

Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
1	SPB	Sample Purification Beads	2° to 8°C
2	SMB	Streptavidin Magnetic Beads	2° to 8°C
1	ET2	Elute Target Buffer 2	2° to 8°C
1	ST	Stop Tagment Buffer	15° to 30°C

Box 2

Quantity	Acronym	Reagent Name	Storage Temperature
1	TDE1	Tagment DNA Enzyme	-15° to -25°C
1	EE1	Enrichment Elution Buffer 1	-15° to -25°C
1	TD	Tagment DNA Buffer	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C
1	NLM	Nextera Library Amplification Mix	-15° to -25°C
1	EHB	Enrichment Hybridization Buffer	-15° to -25°C
1	EWS	Enrichment Wash Solution	-15° to -25°C
1	HP3	2N NaOH	-15° to -25°C
1	PPC	PCR Primer Cocktail	-15° to -25°C
2	NEM	Nextera Enrichment Amplification Mix	-15° to -25°C

Box 3

Quantity	Reagent Name	Storage Temperature
1 tube	Index Primer, E501	-15° to -25°C
4 tubes	Index Primer, N701 to N704	-15° to -25°C

TruSight Rapid Capture Kit Contents (24 Indices, 48 Samples) (FC-140-1104, TG-140-1104)

Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
2	SPB	Sample Purification Beads	2° to 8°C
2	SMB	Streptavidin Magnetic Beads	2° to 8°C
1	ET2	Elute Target Buffer 2	2° to 8°C
1	ST	Stop Tagment Buffer	15° to 30°C

Box 2

Quantity	Acronym	Reagent Name	Storage Temperature
2	TDE1	Tagment DNA Enzyme	-15° to -25°C
1	EE1	Enrichment Elution Buffer 1	-15° to -25°C
1	TD	Tagment DNA Buffer	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C
2	NLM	Nextera Library Amplification Mix	-15° to -25°C
1	EHB	Enrichment Hybridization Buffer	-15° to -25°C
1	EWS	Enrichment Wash Solution	-15° to -25°C
1	HP3	2N NaOH	-15° to -25°C
1	PPC	PCR Primer Cocktail	-15° to -25°C
2	NEM	Nextera Enrichment Amplification Mix	-15° to -25°C

Box 3

Quantity	Reagent Name	Storage Temperature
2 tubes	Index Primer, E501 to E502	-15° to -25°C
12 tubes	Index Primers, N701 to N712	-15° to -25°C

TruSight Rapid Capture Kit Contents (24 Indices, 96 Samples) (FC-140-1105, TG-140-1105)

Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
4	SPB	Sample Purification Beads	2° to 8°C
4	SMB	Streptavidin Magnetic Beads	2° to 8°C
1	ET2	Elute Target Buffer 2	2° to 8°C
1	ST	Stop Tagment Buffer	15° to 30°C

Box 2

Quantity	Acronym	Reagent Name	Storage Temperature
4	TDE1	Tagment DNA Enzyme	-15° to -25°C
1	EE1	Enrichment Elution Buffer 1	-15° to -25°C
2	TD	Tagment DNA Buffer	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C
4	NLM	Nextera Library Amplification Mix	-15° to -25°C
2	EHB	Enrichment Hybridization Buffer	-15° to -25°C
2	EWS	Enrichment Wash Solution	-15° to -25°C
1	HP3	2N NaOH	-15° to -25°C
1	PPC	PCR Primer Cocktail	-15° to -25°C
4	NEM	Nextera Enrichment Amplification Mix	-15° to -25°C

Box 3

Quantity	Reagent Name	Storage Temperature
2 tubes	Index Primer, E501 to E502	-15° to -25°C
12 tubes	Index Primers, N701 to N712	-15° to -25°C

TruSight Rapid Capture Kit Contents (96 Indices, 288 Samples) (FC-140-1106, TG-140-1106)

Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
12	SPB	Sample Purification Beads	2° to 8°C
12	SMB	Streptavidin Magnetic Beads	2° to 8°C
3	ET2	Elute Target Buffer 2	2° to 8°C
3	ST	Stop Tagment Buffer	15° to 30°C

Box 2

Quantity	Acronym	Reagent Name	Storage Temperature
12	TDE1	Tagment DNA Enzyme	-15° to -25°C
3	EE1	Enrichment Elution Buffer 1	-15° to -25°C
6	TD	Tagment DNA Buffer	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C
12	NLM	Nextera Library Amplification Mix	-15° to -25°C
6	EHB	Enrichment Hybridization Buffer	-15° to -25°C
6	EWS	Enrichment Wash Solution	-15° to -25°C
3	HP3	2N NaOH	-15° to -25°C
3	PPC	PCR Primer Cocktail	-15° to -25°C
12	NEM	Nextera Enrichment Amplification Mix	-15° to -25°C

Box 3

Quantity	Reagent Name	Storage Temperature
8 tubes	Index Primer, E501 to E508	-15° to -25°C
12 tubes	Index Primers, N701 to N712	-15° to -25°C

Consumables and Equipment

Check to ensure that you have all of the necessary user-supplied consumables and equipment before proceeding to the sample preparation and enrichment procedures.

NOTE
The TruSight Rapid Capture protocol has been optimized and validated using the items listed below. Comparable performance is not guaranteed when using alternate consumables and equipment.

Table 4 User-Supplied Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes	General lab supplier
20 µl barrier pipette tips	General lab supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
Adhesive seal roller	General lab supplier
96-well flat clear bottom black microplates Note: Used when quantifying samples with a SpectraMax M5 spectrofluorometer.	Corning, part # 3904
96-well storage plates, round well, 0.8 ml (“MIDI” plate)	Fisher Scientific, part # AB-0859

Consumable	Supplier
Hard-Shell 96-well PCR Plates (“HSP” plate)	Bio-Rad, part # HSP-9601
Aluminum foil	General lab supplier
[Optional] Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) Note: Used to concentrate a pooled library. Another option is to use a vacuum concentrator.	Millipore, part # UFC503008
Conical centrifuge tubes (15 ml or 50 ml)	General lab supplier
Distilled water	General lab supplier
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma Aldrich, part # E7023
Microseal ‘A’ film	BioRad, part # MSA-5001
Microseal ‘B’ adhesive seals	BioRad, part # MSB-1001
RNase/DNase-free 8-well PCR strip tubes and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
Tris-Cl 10 mM, pH 8.5	General lab supplier
[Optional] TruSeq Index Plate Fixture Kit Note: Recommended for setting up indexed PCR primers. This part is reusable.	Illumina, catalog # FC-130-1005
Ultra pure water	General lab supplier

Table 5 User-Supplied Equipment

Equipment	Supplier
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA

Equipment	Supplier
[Optional] DNA 1000 Chip	Agilent, part # 5067-1504
[Optional] High Sensitivity DNA Chip	Agilent, part # 4067-4626
DNA Engine Multi-Bay Thermal Cycler See *Thermal Cyclers* on page 63.	Bio-Rad, part # PTC-0240G or PTC-0220G, with Alpha Unit, part # ALS-1296GC
High Speed Micro Plate Shaker	VWR, catalog # • 13500-890 (110V/120V) or • 14216-214 (230V)
Magnetic stand-96	Life Technologies, part # AM10027
Microcentrifuge	General lab supplier
Microplate centrifuge	General lab supplier
MIDI plate insert for heating system	Illumina, catalog # BD-60-601
QuantiFluor dsDNA System or similar fluorometric-based DNA quantification system	Promega, catalog # E2670
SpectraMax M5 spectrofluorometer or similar fluorometric-based DNA quantification system	Molecular Devices, part # 0112-0159
Tru Temp Microheating System	Illumina, catalog # • SC-60-503 (115V) or • SC-60-504 (220V)
[Optional] Vacuum concentrator Note: Used to concentrate a pooled library. Another option is to use Amicon Ultra-0.5 centrifugal filter units.	General lab supplier
Vortexer	General lab supplier

Thermal Cyclers

The following table lists the recommended settings for the Illumina recommended thermal cycler, as well as other comparable models. Validate any thermal cyclers not listed below if your lab has not yet performed the TruSight Rapid Capture protocol.

Thermal Cycler	Temp Mode	Lid Temp	Vessel Type
Bio-Rad DNA Engine Tetrad 2	Calculated	Heated, Constant at 100°C	Polypropylene plates and tubes
MJ Research DNA Engine Tetrad	Calculated	Heated	Plate
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	Plate

Index Sequences

The following list of index sequences is provided for generating sample sheets to demultiplex the samples. A dual indexing strategy uses two 8 base indices, Index 1 (i7) adjacent to the P7 sequence, and Index 2 (i5) adjacent to the P5 sequence. Dual indexing is enabled by adding a unique Index 1 (i7) and Index 2 (i5) to each sample from 12 different Index 1 (i7) adapters (N701–N712) and two different Index 2 (i5) adapters (E501 and E502). In the Index adapter name, the N refers to Nextera, the E refers to enrichment, 7 or 5 refers to Index 1 (i7) or Index 2 (i5), respectively, and 01–12 refers to the Index number.

Table 6 TruSight Rapid Capture Index Adapter Sequences

Index 1 (i7)	Sequence	Index 2 (i5)	Sequence
N701	TAAGGCGA	E501	TAGATCGC
N702	CGTACTAG	E502	CTCTCTAT
N703	AGGCAGAA		
N704	TCCTGAGC		
N705	GGACTCCT		
N706	TAGGCATG		
N707	CTCTCTAC		
N708	CAGAGAGG		
N709	GCTACGCT		
N710	CGAGGCTG		
N711	AAGAGGCA		
N712	GTAGAGGA		

NOTE

While the E500 series Index 2 (i5) sequences in the TruSight Rapid Capture kits are identical to S500 series Index 2 (i5) sequences in other kits, the Index 2 (i5) adapters are not interchangeable across kits.

Low-Plex Pooling Guidelines

Follow these guidelines when pooling less than 6 libraries. Illumina uses a green laser to sequence G/T and a red laser to sequence A/C. At each cycle at least one of two nucleotides for each color channel need to be read to ensure proper registration. It is important to maintain color balance for each base of the index read being sequenced, otherwise index read sequencing could fail due to registration failure. Follow the instructions described here to determine which libraries are pooled pre-enrichment.

The TruSight Rapid Capture kits support pre-enrichment pooling of up to 12 different indexed samples. Illumina recommends the following:

- For pooling 12 samples pre-enrichment, pool samples with Index 1 (i7) 701–712 (any Index 2 (i5)), followed by a single-index sequencing run.
- For pooling <12 samples, set up a single index workflow sequencing run using different Index 1 (i7) indices (any Index 2 (i5)). Illumina provides compatible i5 indices in this kit, but when pooling less than 6 i7 indices use the combinations in Table 7 for proper color balancing.
- See Table 8 for pooling details when using both Index 1 (i7) and Index 2 (i5), followed by a dual indexed sequencing run.

NOTE

Illumina provides enough reagents to pool 12 samples at a time. If you choose to pool less than 12 samples during the enrichment, the reagents provided in your kit may not support the number of samples as noted on the kit box.

NOTE

There is sufficient volume in each Index 1 (i7) tube for 16 reactions and in each Index 2 (i5) tube for 24 reactions. Plan your experiment accordingly so you do not to run out of index primers.

Table 7 Libraries Pooled: 6 or Fewer; Sequencing Workflow: Single Index

Plex	Index 1 (i7) Selection	Index 2 (i5) Selection
1-plex (no pooling)	Any Index 1 adapter	Any Index 2 adapter
2-plex	• [option 1] N702 and N701 • [option 2] N702 and N704	
3-plex	• [option 1] N701, N702, and N704 • [option 2] N703, N705, and N706	
4- or 5-plex	• [option 1] N701, N702, N704, and any other Index 1 adapter • [option 2] N703, N705, N706, and any other Index 1 adapter	
6-plex	N701, N702, N703, N704, N705, and N706	

Table 8 Sequencing Workflow: Single or Dual Index

Plex	Index 1 (i7) Selection	Index 2 (i5) Selection
7–12 plex, Dual Index	• [option 1] N701, N702, N704, and any other Index 1 adapter (as needed) • [option 2] N703, N705, N706, and any other Index 1 adapter (as needed)	E501 and E502
7–12 plex, Single Index	• N701–N706 and any other Index 1 adapter (as needed)	Any Index 2 (i5) adapter

The table below is an example of the acceptable combinations for four pooled samples. Alternatively, please check the real sequences of each index in Table 8 to make sure each base position will have signal in both color channels for the index read. Both i5 indices (E501 and E502) are compatible with each other

Sample	Good Index 1		Bad Index 1	
1	701	TAAGGCGA	702	CGTACTAG
2	702	CGTACTAG	703	AGGCAGAA
3	704	TCCTGAGC	711	AAGAGGCA
		√ √ √ √ √ √ √ √		x √ x x √ x x √

√=signal in both color
x=signal missing in one color channel

DNA Quantitation

Illumina recommends the QuantiFluor dsDNA assay to quantitate dsDNA samples, because it can quantitate small DNA volumes and measures DNA directly. Other techniques may pick up contamination such as RNA and proteins. Illumina recommends using a spectrofluorometer, because fluorometry provides DNA-specific quantification. Spectrophotometry might also measure RNA and yield values that are too high.

Consumables

Item	Quantity	Storage	Supplied By
1X TE	as needed	15° to 30°C	User
96-well flat clear bottom black microplates	2 per 96 samples	15° to 30°C	User
96-well MIDI plates	2 per 96 samples	15° to 30°C	User
Aluminum foil	as needed	15° to 30°C	User
Conical centrifuge tube (15 ml or 50 ml)	1	15° to 30°C	User
Lambda DNA	as needed	2° to 8°C	User
Microseal 'B' adhesive seals	1 per microplate + 1 per MIDI plate	15° to 30°C	User
QuantiFluor dsDNA dye	as needed	2° to 8°C	User
RNase/DNase-free Reagent Reservoir	1	15° to 30°C	User

Preparation

- Remove the QuantiFluor dsDNA dye from to 2° to 8°C and let stand at room temperature for 60 minutes in a light-impermeable container.
- Label a new 96-well MIDI plate **Lambda DNA Stock** with a smudge resistant pen.
- Label a new 96-well MIDI plate **DNA Stock** with a smudge resistant pen.
- Label a new 96-well microplate **Lambda DNA Quant** with a smudge resistant pen.

Make Lambda DNA Stock Plate

1 Add stock Lambda DNA to well A1 in the MIDI plate labeled Lambda DNA Stock and dilute it to 1 ng/µl in a final volume of 300 µl. Pipette up and down several times.

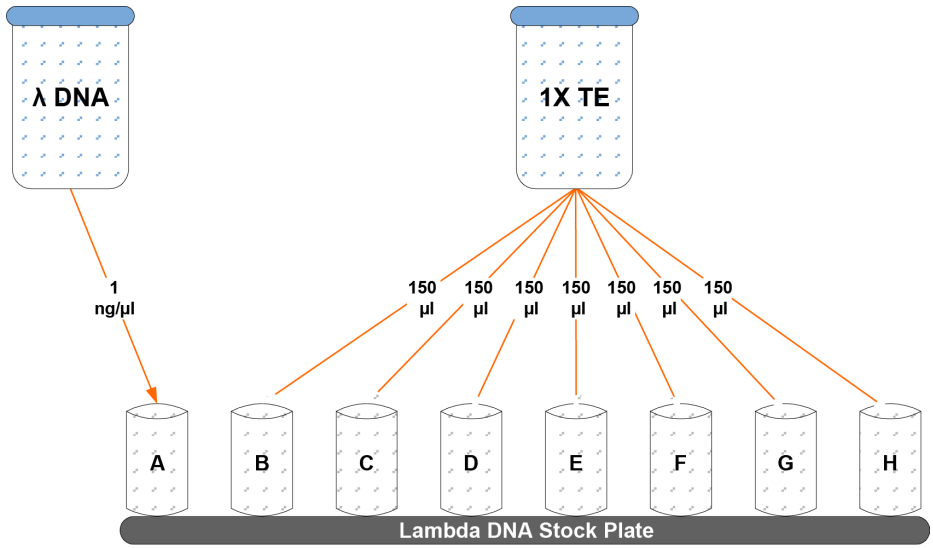
a Use the following formula to calculate the amount of stock Lambda DNA to add to A1:

$$\frac{(300\ \mu l) \times (1\ ng/\mu l)}{\text{(stock Lambda DNA concentration)}} = \mu l \text{ of stock Lambda DNA to add to A1}$$

b Dilute the stock DNA in well A1 using the following formula:
(300 µl) - (µl of stock Lambda DNA in well A1) = µl of 1X TE to add to A1

2 Add 150 µl 1X TE to well B, C, D, E, F, G, and H of column 1 of the Lambda DNA Stock plate.

Figure 6 Dilution of Stock Lambda DNA Standard



3 Transfer 150 µl of Lambda DNA from well A1 into well B1. Pipette up and down several times to mix.

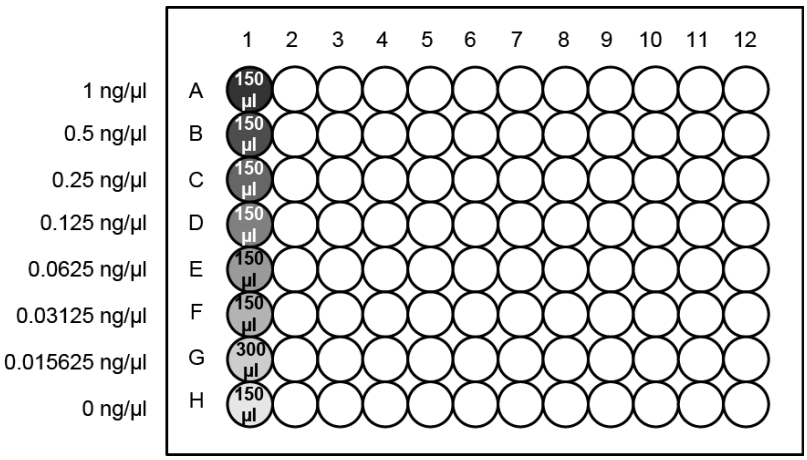
4 Transfer 150 µl of liquid from well B1 into well C1. Pipette up and down several times to mix.

5 Repeat the transfer for wells D1, E1, F1, and G1, changing tips each time. **Do not transfer from well G1 to H1.**Well H1 serves as the blank 0 ng/µl Lambda DNA.

Table 9 Concentrations of Lambda DNA

Row-Column	Concentration (ng/µl)	Final Volume in Well (µl)
A1	1	150
B1	0.5	150
C1	0.25	150
D1	0.125	150
E1	0.0625	150
F1	0.03125	150
G1	0.015625	300
H1	0	150

Figure 7 Serial Dilutions of Lambda DNA



6 Seal the Lambda DNA Stock plate with a Microseal 'B' adhesive seal.

Make DNA Stock Plate

1 In the MIDI plate labeled DNA Stock, prepare the appropriate dilutions of your DNA samples using 1X TE. For accuracy, Illumina recommends measuring each sample in triplicate. Make sure at least 50 µl of diluted sample is prepared for quantification with the QuantiFluor dsDNA dye. If performing replicate measurements, scale appropriately. Illumina recommends the following dilution, depending on the DNA sample. This dilution may need to be adjusted depending on sample quality. Do one of the following:
   - For high quality gDNA, dilute your samples 1:1000. For example: 2 µl of gDNA + 1998 µl of 1X TE.
   - For pre-enriched Nextera libraries, dilute your libraries 1:200. For example: 2 µl of library sample + 398 µl of 1X TE.
   - For post-enriched Nextera 12-plex exome capture libraries, Illumina recommends a 1:100 dilution. For example: 2 µl of post-enriched library + 198 µl of 1X TE.
   - For post-enriched Nextera custom capture libraries, the appropriate dilution is based on total targeted region size and the level of indexing.

2 Mix thoroughly as follows:
   a Cover the DNA Stock plate with a lid.
   b Shake the DNA Stock plate on a microplate shaker at 1200 rpm for 1 minute.

3 Centrifuge the DNA Stock plate to 280 xg for 1 minute

Dilute QuantiFluor dsDNA Dye

1 Prepare a 1:200 dilution of QuantiFluor dsDNA dye into 1X TE using a conical centrifuge tube wrapped in aluminum foil.
For accuracy, Illumina recommends running each sample and standard in triplicate. For each measurement 40 µl of diluted QuantiFluor dye is required. Scale as appropriate.

NOTE
Quantifluor dsDNA dye is often still crystallized at room temperature. Before use, visually verify that the dye is fully thawed and liquid.

2 Cap the foil-wrapped tube and vortex to mix.

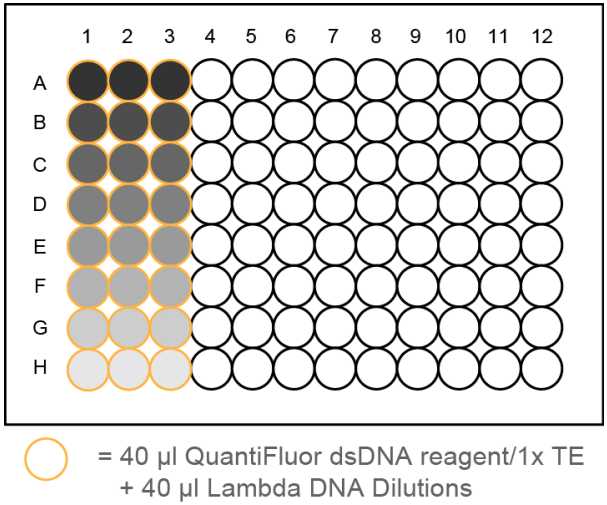
Make Lambda DNA Quant Plate

1 Pour the diluted QuantiFluor dsDNA dye/1X TE into a clean reagent reservoir.

2 Using a multi-channel pipette, transfer 40 µl of diluted QuantiFluor dsDNA dye/1X TE into each well of columns 1–3 of the microplate labeled Lambda DNA Quant.

3 Transfer 40 µl of each stock Lambda DNA dilution from the Lambda DNA Stock plate to columns 1–3 of the Lambda DNA Quant plate.

Figure 8 Lambda DNA Quant Plate with QuantiFluor dsDNA Dye/1X TE



4 Mix thoroughly as follows:
   a Cover the Lambda DNA Quant plate with a lid.
   b Shake the Lambda DNA Quant plate on a microplate shaker at 1200 rpm for 1 minute.

5 Centrifuge the Lambda DNA Quant plate to 280 xg for 1 minute

6 Protect the Lambda DNA Quant plate from light until it is read by the spectrofluorometer.

Make DNA Quant Plate

1. Using a multichannel pipette, transfer 40 µl QuantiFluor dsDNA reagent/1X TE dilution to each well of the microplate labeled DNA Quant that will contain a sample.
2. Remove the lid from the DNA Stock plate
3. Transfer 40 µl of each DNA sample in the DNA Stock plate to the corresponding well of the DNA Quant plate containing QuantiFluor dsDNA reagent/1X TE.
4. Mix thoroughly as follows:
   a. Cover the DNA Quant plate with a lid.
   b. Shake the DNA Quant plate on a microplate shaker at 1200 rpm for 1 minute.
5. Centrifuge the DNA Quant plate to 280 xg for 1 minute
6. Protect the DNA Quant plate from light until it is read by the spectrofluorometer.

Read Quantitation Plate

1. Turn on the spectrofluorometer.
2. At the PC, open the SoftMax Pro program.
3. Measure fluorescence (485 nm Ex / 538 nm Em) of both the Lambda DNA Quant and DNA Quant plates according to the spectrofluorometer/software recommendations.
4. Calculate the DNA concentration of your unknown samples using the fluorescence values determined from step as follows:
   a. Calculate the average relative fluorescence units (RFU) of the Lambda DNA standards run in triplicate on the Lambda DNA Quant plate.
   b. Calculate an Adjusted RFU by subtracting the RFU of the blank Lambda DNA standard (0 ng/µl) Row H from all unknown and standard samples.
   c. Create a scatter plot of the Lambda DNA standard curve values with the Adjusted RFU on the Y axis and DNA concentration (ng/µl) on the X axis.
   d. Determine the equation of the line for the Lambda DNA standard curve values which will be in the format of y = mx + b is equivalent to RFU = (slope*concentration) + y_int.
   e. Calculate the concentration for each unknown sample by using the RFU for each sample for y in the equation and determining the value for x in ng/µl.

f Multiply the resulting concentration by the appropriate dilution factor.

g Use the following formula to convert from ng/µl to nM. Assume a 400 bp library size or calculate based on the average size (insert + adapters) of the enriched library as determined by a Agilent Technologies 2100 Bioanalyzer trace:

$$\frac{\text{(concentration in ng/µl)}}{\text{(660 g/mol *average library size)}} \times 10^6 = \text{concentration in nM}$$

For example:

$$\frac{\text{15 ng/µl)}}{\text{(660 g/mol *400)}} \times 10^6 = 57 \text{ nM}$$

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 10 Illumina General Contact Information

Illumina Website	www.illumina.com
Email	techsupport@illumina.com

Table 11 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at www.illumina.com/msds.

Product Documentation

Product documentation in PDF is available for download from the Illumina website. Go to www.illumina.com/support, select a product, then click **Documentation & Literature**.

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